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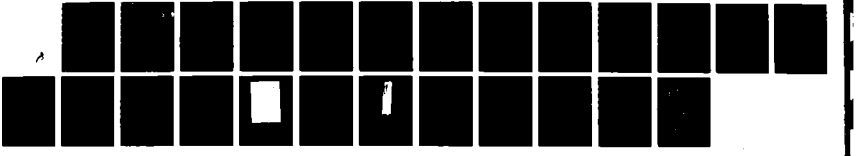

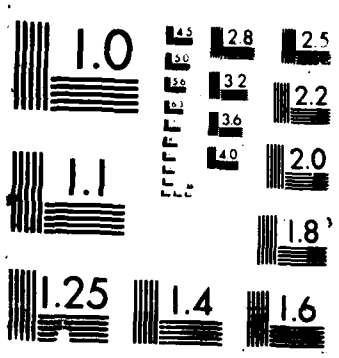
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It has been known for many years that stressful situations can be a contributing factor in the development of a variety of bacterial, viral and neoplastic diseases. Specifically, decreased immuno-competence seems to account for the increased susceptibility of stressed hosts for these disease states. We have suggested that one mechanism by which this can occur is through the action of neuroendocrine hormones. It appears that the immune and neuroendocrine systems communicate in a bidirectional regulatory circuit by virtue of common signal molecules and receptors. In this report we discuss our findings on the amino acid sequence and post translational processing of lymphocyte derived corticotropin (ACTH). Also, presented are results that the hypothalamic releasing hormones for luteinizing hormone (LH) and thyrotropin (TSH) induce lymphocytes to synthesize immunoreactive LH and TSH, respectively. Finally, we discuss our data that the ACTH receptor on lymphocytes acts through adenylate cyclase like the prototype on adrenal tissue.

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22a NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Dr. J.A. Majde	(202) 696-4055	ONR

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I. Background

It has been suggested for many years that stressful situations can be a contributing factor in an individual's resistance to infections and tumors. We and others have shown that one mechanism by which this can occur is through the action of neuroendocrine hormones on the immune system (1,2). In addition, we have found that lymphocytes synthesize biologically active molecules identical to neuroendocrine hormones. Thus, both arms of a regulatory circuit between the immune and neuroendocrine systems exist and could provide bi-directional communication between the two systems. The significance of this relationship is just beginning to be determined, but initially it appears to be a mechanism whereby behavior and stress can enhance susceptibility to disease or affect healing. Thus, if true, the implications would be major, especially for the military. Once understood, it may be possible to block this stress effect, thereby preventing disease and the subsequent inefficiency or disruption of training and other activities.

II. Specific Aims

The overall objective of the project is to characterize the molecules and mechanisms by which the immune and neuroendocrine systems interact. In particular, this project is aimed at determining if the hypothalamus can modulate immune responses directly by hypothalamic hormones or indirectly through activation of other neuroendocrine tissues. More specifically the aims of the original project include:

1. Characterization of lymphocyte immunoreactive corticotropin (ir-ACTH) induced by corticotropin releasing factor (CRF).

2. To determine if CRF induces lymphocytes to make immunoreactive (ir) endorphins.

3. To determine if other hypothalamic releasing factors (RF) stimulate ir hormone production by lymphocytes.

4. Characterization of RF immunomodulatory activity.

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III. Results

A. Amino Acid Sequencing of Lymphocyte-derived ACTH

Previously we have reported that the hypothalamic releasing hormone, corticotropin releasing factor (CRF), induced lymphocytes to produce a molecule very similar, if not identical to corticotropin (ACTH) (3). Bacterial lipopolysaccharide (LPS) or endotoxin also induced lymphocyte ACTH production (4) although it is processed to a shorter species of ACTH (5). We have utilized this latter system to definitively prove that the

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lymphocyte-derived ACTH is identical with the pituitary product by amino acid sequencing the lymphocyte material.

Splenocytes from fifty C3H/FeJ mice yielded approximately 7 nmoles of homogenous immunoreactive (ir) ACTH. One-third of the purified ir ACTH corresponded to the full length molecule (4.5 Kd) and the rest to a processed shorter species migrating by HPLC similar to $ACTH_{1-24}$ (Fig. 1). The lower molecular weight species had an amino acid sequence identical to ACTH (Fig. 2). After the first 15 residues the signal faded such that only a few strong amino acids were detected between residues 15 and 25. Since no residues past portions 25 were detected the processed species is no more than 25 amino acids in length and may be shorter based on the weak signal at the carboxy terminus. The amino acid sequence of the larger species suggests it is the full 39 amino acids in length and only one amino acid at position 31. At this residue we found a leu instead of ser (6). Conceivably this could be due to single incorrect base in the cAMP sequence. Other than this the leukocyte ACTH is identical to the prototype molecule supporting the concept that the same proopiomelanocortin gene is expressed in both types of cells.

B. Luteinizing Hormone Releasing hormone (LHRH) Inductions of ir LH in Lymphocytes

Since CRF is able to induce ACTH production by lymphocytes, we have examined other releasing factors for related activities. One factor we have been successful with is LHRH. Treatment of human peripheral blood leukocytes with LHRH (100 ng/ml) for 48 hour induced ir LH production in approximately 35% of the cells as detected through immunofluorescent staining of the cells. The ir LH was synthesized In vivo as radiolabeled amino acids were incorporated into material that bound to a specific anti-LH anti-body affinity column. Some ir LH is produced constitutively nonstimulated cultures. SDS-polyacrylamide gel electrophoresis analysis of the ir LH shows it to consists of two chains migrating identically with the α and β chains of human LH (each approximately 15.5 Kd). Since LH is antigenically related to chorionic gonadotropin (CG) and this is also synthesized by lymphocytes in an MLR (7), we have absorbed our anti-LH antiserum with hCG. This absorption has no effect on our results, which supports our data that lymphocytes synthesize LH. An initial experiment testing for bioactivity was successful. Purified ir LH induced mouse Leydig cells to produce testosterone. Future experiments will characterize the specificity and dose response of this bioactivity, as well as testing LHRH for other immunomodulatory activities.

C. Thyrotropin Releasing Hormone (TRH) Induction of ir TSH in Lymphocytes

Previously we found that T-lymphocyte mitogens such as staphylococcal enterotoxin A (SEA) induced lymphocytes to synthesizes ir TSH (8). Therefore it was logical based on our CRF results to test TRH for the ability to induce ir TSH production by lymphocytes. Treatment of human peripheral blood leukocytes with 50 µg/ml of TRH induced 37% of the cells to stain positive by immunofluorescence for TSH.

We have been able to use this system as a means to examine neuroendocrine dysregulation in major depressive disorder (9). A subgroup of individuals with major depressive disorder had an impaired thyrotropin (TSH) response to thyrotropin releasing hormone (TRH). The molecular relationship between the mechanism of this "blunted" TSH response and depression is unknown. Numerous, recent studies have characterized similarities and interactions between the immune and neuroendocrine systems. Since the immune system both produces and responds to TSH, we utilized a peripheral blood leukocyte system to compare immunoreactive (ir)-TSH responsiveness in 10 adult patients (1M, 9F) with Research Diagnostic Criteria for major depressive disorder to that of 9 control subjects. All subjects had normal baseline serum TSH and T4 concentrations. Isolated mononuclear leukocytes were treated in vitro with either 0.5 µg/ml Staphylococcal enterotoxin A (SEA), 50 µg/ml TRH or no stimulant. After incubation, the cells were monitored for ir-TSH production by indirect immunofluorescence and reverse hemolytic plaque assay using antisera to TSH-β. The culture supernates were analyzed by TSH-radioimmunoassay. SEA and TRH treated cell cultures from depressed individuals had significantly fewer immunofluorescent positive cells as well as significantly fewer number and size of plaques than did similarly treated leukocytes from control subjects. The increase in supernatant ir-TSH was significantly less in TRH treated cultures from depressed patients as compared to normals ($P < .05$). These results suggested that examination of mononuclear leukocyte TSH production may reflect an altered state of neuroendocrine function and thus be a useful marker for major depressive disorder.

D. TRH Induction of ir TSH in Molt 4 Cells

For the purposes of structurally characterizing ir TSH and studying its production, we have examined several continuous cell lines for ir TSH production.

Detection of ir-TSH: Immunofluorescent (IF) analysis was performed on 5 T-lymphoblastic cell lines. Table 1 shows that Molt 4 cells but not other cell lines demonstrated significant IF when stained with antisera to TSH-β. The IF increased with Staphylococcal enterotoxin A (SEA) and TRH thus implying increased production or induction of ir-TSH in the Molt 4 T cells.

Table 1

Indirect immunofluorescence of T cell lines stained with anti-β-TSH antisera.

	Treatment		
ATCC cell line	Mock	SEA	TRH
Molt 4	28 ± 12*	46 ± 10	40 ± 8
Molt 3	11 ± 5	12 ± 7	11 ± 10
Hut 78	6 ± 1	4 ± 2	5 ± 1
CCRF CEM	7 ± 4	5 ± 5	6 ± 3
CCRF HSB-2	10 ± 2	12 ± 7	6 ± 8

* Numbers are expressed as % staining positive with anti TSH-β minus % staining positive with NRS ± S.D.

Reverse hemolytic plaque assay (RHPA), (Figure 3,): As a verification of the IF results, RHPA, again with antisera to TSH-β, was performed on SEA or TRH stimulated Molt 4 cells. Panel A, Figure 1, shows specific plaques or zones of hemolysis surrounding lymphocytes indicating release of ir-TSH from SEA stimulated Molt 4 cells. Panel B is a higher power view showing intact lymphocytes. Panel C is TRH stimulated Molt 4 cells showing that some of the plaques are apparently larger than those around SEA treated cells. Panel D is an antibody control indicating no non-specific lysis. Therefore, we conclude that SEA and TRH treated Molt 4 cells are producing and releasing ir-TSH.

Measurement of ir-TSH production from Molt 4 cells (Table 2): In order to analyze the ir-TSH production in a more quantitative manner, we performed enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) on the culture supernates from the 5 T cell lines. Whereas the ELISA and RIA reactions were below the level of detection for the cells that did not fluoresce significantly (data not shown) from the Molt 4 cells, we detected the production of nanogram (ng) quantities of ir-TSH in both assays. The amount of ir-TSH increased 3 to 8 fold after treatment with SEA or TRH suggesting induction or upregulation of a constitutive ir-TSH production. Affinity column purification and subsequent elution of bound radiolabeled material showed a significant increase in the amount of acid precipitable material in the culture supernates of SEA and TRH treated Molt 4 cells (Table 2). This is strong evidence that the ir-TSH is synthesized de novo.

Table 2

In vitro production of ir-TSH from 5 x 10^7 MOLT 4 cells.

Treatment	ELISA (ng)	RIA (ng)	% Specific incorporation of total protein into ir-TSH
Mock	5 ± 2	3 ± 1	.02
SEA	12 ± 4	10 ± 5	.1
TRH	23 ± 3	24 ± 3	.12

ELISA was performed as described (9). Briefly, pituitary derived TSH obtained from the National Pituitary Institute was coated onto U-bottom plates in order to obtain a standard curve. Culture supernates from variously treated cells were individually diluted 1:2 in coating buffer and plated in guadruplicate across the plate. After attachment, each antigen was reacted with either anti β-TSH or NRS both at a 1:200 dilution. This was followed by incubation with alkaline phosphatase-goat anti rabbit at 1:300 dilution. Substrate was added and absorbance read at 405 nm. Numbers are expected as ng of TSH read from protein plot of standard TSH minus the aborbance with NRS.

*Refers to $\frac{\%\ \text{of acid insoluable cpm in bound material}}{\%\ \text{of acid insoluable cpm in culture supernate}}$

Protein structure of purified ir-TSH: HPLC analysis was performed on radiolabeled, affinity purified ir-TSH. The elution profile of the Molt 4 T cell derived ir-TSH is similar to that seen for pituitary derived I^{125} TSH indicating a similar molecular mass between the two substances. In addition, the affinity purified ir-TSH bound to a concanavalin A (Con A) lectin affinity column (data not shown) indicating that it was partially glycosylated.

Subsequent sizing of the affinity purified ir-TSH was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as shown in Figure 4.

We consistently saw 2 major peaks of radioactivity on reducing gels, approximately 17K and 21K, which could correspond to the β and α TSH subunits, respectively. We have also seen the presence of a larger molecular weight material that migrates at a range of 33 Kd to 52 Kd which may represent intact TSH. In addition, we consistently see larger molecular weight material which may be ir-TSH aggregates.

Therefore, our preliminary results supply evidence that the Molt 4 T cell line produces ir-TSH that is very similar to pituitary TSH based on subunit structure, antigenicity, glycosylation, HPLC, RIA and induction with TRH. A limitation of our biochemical analysis is the lack of amino acid and carbohydrate sequence data for the ir-TSH, however, our proposed studies plan to address this project. In addition, we propose bioactivity studies on thyroid cells as well as immunocytes since pituitary TSH is active in both systems.

mRNA analysis (Figure 5): As a verification of our protein studies, we performed slot blot analysis on total cellular RNA extracts, using a probe specific to the β chain of rat TSH (kindly provided to us by Dr. William Chin). There is an increase in the amount of RNA that is present in SEA and TRH stimulated cells at a 6 hour time point. BCL-1 cells, of B lymphocyte lineage, do not show a significant amount of mRNA for TSH-β in the same amount of total cellular RNA. Figure 5 shows the results of Northern blot analysis. Lanes 1-4 (left to right) represent total RNA from TRH treated Molt 4 cells at 2, 4, 6 and 24 hours, respectively. A small mRNA species begins to appear at 4 hours, is stronger at 6 hours and appears to be reduced at 24 hours. The results of these experiments provide very strong evidence that the TSH β gene is being expressed in Molt 4 cells and in addition strengthens our results characterizing the protein structure.

Therefore this biochemical and molecular evidence provides rationale for using the Molt 4 T cell as a model for exploring mechanisms of regulation of ir-TSH that are unique as well as common to immune-neuroendocrine system pathways. This type of evidence would be important in documenting the molecular basis for interactions between the two systems and substantiating a holistic approach to mental health. In addition, the Molt 4 cells may be used as a standard cell control for our proposed studies with leukocytes from MDD patients.

TRH receptor binding studies (Figure 6): We reasoned that since the Molt 4 cells increased ir-TSH production after treatment with TRH and SEA, these cells may have TRH receptors. We performed radioligand binding studies on intact Molt 4 cells with radiolabeled TRH. Figure 6 represents a Scatchard analysis and resolution of two binding sites, a high affinity site with Kd approximately 4.1×10^{-11}M with approximately 400 sites/cell and a lower affinity site with a Kd of approximately 1.8×10^{-8}M. The higher affinity site is saturable and the binding of radiolabeled TRH is specific since excess cold TRH inhibits binding. The lower affinity site is similar to what has been reported in the pituitary gland. Thus, analysis of this receptor with regards to kinetics, Bmax, regulation of expression, functionality and coupling to second messengers may serve as a model for the pituitary TRH receptor. The higher affinity TRH receptor has not been reported in the pituitary gland and certainly function studies are imperative from a basic science standpoint as we may be measuring a TRH receptor unique to the immune system. The high affinity receptor provides a mechanism whereby the cells of the immune system could respond to microenvironmental or peripheral physiologic TRH concentrations. Competitive binding analyses with cold TRH and SEA, show that the binding is specific as cold TRH competes for binding sites. Conversely, SEA does not compete for binding and obviously does not bind to TRH receptors on the Molt 4 cells. These results imply that SEA and TRH induce ir-TSH through different extracellular receptors and possibly through different intracellular mechanisms. These findings suggest that Molt 4 cells provide a unique model system for studying TRH induction of the TSH genes.

E. Lymphocyte ACTH Production in Major Depressive Disorder (MDD)

Lymphocytes from controls or MDD patients were treated in vitro with Newcastle disease virus (NDV), CRF and vasopressin (AVP), with or without dexamethasone (DEX). The cultures were harvested and the cells stained by immunofluorescence for ACTH. The MOCK, NDV and NDV + DEX results were

analyzed using multivariate ANOVA with repeated measures for the MDD and control groups. When all three experimental conditions were considered together, leukocyte ACTH production was higher in the MDD group compared to the control group (p=.006). Each experimental condition differed significantly from the others (p=.04). ACTH production was higher in the MDD group than the control group for all of the three types of experimental conditions. Table 2 shows the p values for each comparison. The difference was most marked when the cells were exposed to NDV (p = .0045) and to NDV + DEX (p = .002). [An outlier with high ACTH production in the NDV + DEX was removed from the analysis because of rejection of the normality hypothesis in the total sample. With this patient included p is .013. The means reported in Table 2 include the outlier.] In the NDV + DEX experiment the mean level of % fluorescence in the MDD group was double that of the controls. Also in the presence of DEX, about two-thirds of the MDD patients failed to block ACTH production to below the MOCK level compared to one third of the control group in the same experiment. The trend towards failure of adequate suppression of ACTH production by dex is further brought out by using a low arbitrary cutoff of 15 % positive immunofluorescence in the NDV + DEX as an indication of adequate suppression: only 30% of depressed patients showed % cell fluorescence below 15% compared to 75% of the control group, (Fisher exact test, p = .011).

Multivariate ANOVA, with repeated measures, showed a statistically significant difference between the MDD group and the control group (p=.05) for the MOCK, CRH + AVP and CRH + AVP + DEX. The difference between the different experimental condition terms were not statistically different. CRH + AVP also stimulated ACTH production more in the MN-WBC from MDD patients than those from the controls, (p=.031), but the difference between the groups for the CRH + AVP + DEX test was not significant, see Table 3.

Table 3

CONTROL DAY MN-WBC ACTH STIMULATION STUDIES

TEST	MDD GROUP		CONTROL GROUP		
	N	Mean ± Std Dev % Cells immunoflorescence positive for ACTH	N	Mean ± Std Dev % Cells immunoflorescence positive for ACTH	SIGNIF p
MOCK	17	20.9 ± 12.9	13	15.1 ± 8.3	NS*
NDV	18	27.8 ± 11.9	13	16.8 ± 8.1	.0045
NDV + DEX	18	22.3 ± 13.4	13	11.0 ± 10.5	.002
CRH + AVP	14	29.5 ± 15.1	12	18.8 ± 7.7	.031
CRH + AVP + DEX	11	23.2 ± 11.1	12	19.0 ± 8.0	NS

* NS = not significantly different

Thus there are statistically significant differences seen in lymphocyte ACTH production with MDD versus control lymphocytes. Future experiments are aimed at determining the biological significance and mechanisms of the effect.

F. Bacterial Lipopolysaccharide (LPS) Induction of Proopiomelanocortin (POMC)

One of our findings from this ONR contract was that LPS would induce both human and mouse ymphocytes to produce ACTH and endorphins, cleavage products of POMC. Following up this finding, we have characterized the production, processing, and action of this products in LPS, induced shock. Abstracts of these studies are provided below.

Splenic Lymphocyte Production of an Endorphin during Endotoxic Shock (Publication #3).

Endogenous opioids have been reported to elicit some of the pathophysiologic responses to endotoxic shock by binding to the δ-opiate receptor. We have previously reported the production of immunoreactive (ir)-endorphin by B lymphocytes treated with bacterial lipopolysaccharide (LPS). We postulated that this lymphocyte-derived ir-endorphin may be an extrapituitary source of the endogenous opioid component associated with the pathophysiology of endotoxic shock. To test this hypothesis, we chose to study the LPS-sensitive (C3HeB/FeJ) and -resistant (C3H/HeJ) inbred mouse model. We treated these mice with intraperitoneal injections of LPS or B-lymphocyte-derived ir-endorphin. The LPS-sensitive mice presented with a severe hypothermic and pathophysiologic response pattern when treated with LPS or with ir-endorphin. The LPS-resistant mice, which were unresponsive to the LPS, however, presented with the typical hypothermic and pathophysiologic responses to the ir-endorphin. Immunofluorescence on the splenic leukocytes in the LPS-treated mice showed significant ir-endorphin present only the LPS-sensitive mice at a time point preceding onset of the pathophysiologic response pattern. Taken together, this evidence strongly suggests a role for B-lymphocyte-derived ir-endorphin in the pathophysiology of endotoxic shock. The implications of immune system regulation of neuroendocrine function are discussed.

Novel Processing Pathway for Proopiomelanocortin in Lymphocytes: Endotoxin Induction of a New Prohormone-cleaving Enzyme (Publication #7).

We have discovered that the immune system processes proopiomelanocortin (POMC) products differently depending on the stimulus for induction. We have shown that corticotropin releasing factor (CRF) induces the lymphocytes from C3HeB/FeJ (LPS sensitive) mice to produce adrenocorticotropin (ACTH) 1-39 and β-endorphin, whereas LPS induces these lymphocytes to produce ACTH 1-23 to 26 and α- or γ -endorphin. We have proposed that the smaller species of ACTH and endorphin are proteolytic cleavage products from ACTH 1-39 and β-endorphin. Analysis of C3HeB/FeJ LPS treated B lymphocyte lysates showed an enzymatic activity at pH 5 but not pH 7 which cleaved ACTH 1-39 into a smaller ACTH 1-23 to 26. The B lymphocytes from C3H/HeJ (LPS resistant) mice expressed but did not process pro-opiomelanocortin after LPS or CRF treatment nor did their B cells express the aforementioned enzymatic activity. Taken together, these data suggest a unique processing pathway in LPS treated B lymphocytes and one in which ir-endorphins may play a role in the pathophysiology of endotoxic shock.

G. Characterizations of ACTH Receptors on Lymphocytes

One of our specific aims has been to characterize lymphocyte ACTH receptors for structural and functional similarities to the prototype receptor on adrenal tissue. Described below are the abstracts of two studies on lymphoid ACTH receptors.

An ACTH Receptor on Human Mononuclear Leukocytes: Relation to Adrenal ACTH-receptor Activity (Publication #6).

Human peripheral mononuclear leukocytes from normal individuals were shown to have high (Kd=0.04 nM) and lower (Kd=3.4 nM) affinity surface receptors for corticotropin (ACTH). There were approximately 2000 high and 41,600 lower affinity binding sites, per average leukocyte. ^{125}I-ACTH bound specifically and rapidly to both receptors on intact cells. Conversely, lymphocytes from an individual with ACTH insensitivity syndrome demonstrated neither receptor. Thus, the leukocyte ACTH receptor correlates with the supposedly defective adrenal ACTH receptor in this syndrome. The ability to use leukocytes for diagnostic and characterization purposes when the prototype receptors are inaccessible is discussed.

Presence of ACTH and its Receptors on a B Lymphocytic Cell Line: A Possible Autocrine Function for a Neuroendocrine Hormone (Publication #2).

A mouse B lymphocytic cell line, designated BCL_1, was found to produce immunoreactive ACTH and to secrete this molecule into culture supernates. The BCL_1-derived ACTH induced Y-1 adrenal cells to undergo a steroidogeneic response and was eluted from gel filtration columns at a molecular weight similar to that expected for pituitary-derived ACTH. Furthermore, ACTH receptors were detected on the surface of BCL cells using indirect immunofluorescence and ^{125}I-ACTH binding studies. Scatchard analysis demonstrated the presence of high and low affinity binding sites with dissociation constants of 4.5×10^{-12} M and 2.8×10^{-10} M, respectively. The production of both ACTH and its receptor by this B lymphocyte cell line suggests that an autocrine mechanism might be important for the maintenance of the transformed phenotype.

Most recently, we have begun to address the intracellular events triggered by ACTH. Because ACTH has been shown to induce cyclic AMP (cAMP) production in adrenal cells as a component of steroidogeneic response, it is important to determine whether ACTH induces the same intracellular signal in leukocytes. In addition, involvement of cAMP in a variety of leukocyte functions has been demonstrated; immune responses have been shown to be inhibited or augmented, depending upon the function analyzed (reviewed in 1,2). An assay to measure cAMP in leukocytes has been established and the results of two preliminary experiments are shown in Table 4. Both forskolin (a potent activator of adenylate cyclase) and ACTH induce comparable increases in leukocyte cAMP. Because spleen cell populations consist of several types (primarily B cells, T cells, and macrophages), the effect of ACTH in terms of cAMP production will be tested for individual cell types as well. Cell lines representative of B cell, T cell, and macrophage populations will be used in these experiments. Other investigations will determine time- and ACTH concentration-dependence of the response.

Table 4

Induction of cAMP production by forskolin and ACTH

Treatment	picomole cAMP/106 cells
Experiment 1 (Molt 4b cells)	
media (1 minute)	2.8
media (1 hour)	4.3
forskolin (1 minute)	74.1
forskolin (1 hour)	128.0
Experiment 2 (normal C57 B1/6 spleen cells)	
media (1 minute)	0.18
media (1 hour)	3.2
ACTH (1 minutes)	52.3
ACTH (20 minutes)	61.2

[a]Molt 4b lymphoblasts or mouse splenocytes were incubated for the times shown in the presence or absence of forskolin (10-5M) or ACTH (10-5M). Cells were centrifuged and reactions stopped by addition of tricholoracetic acid to cell pellets. Supernates were extracted with ether and cAMP was quantified by a competitive binding assay.

IV. Publications (Year 1 of renewal):
(Copies will follow under separate cover and be distributed as Technical Reports)

1. Harbour-McMenamin, D.V., E.M. Smith and J.E. Blalock. 1986. Production of immunoreactive gonadotropin in mixed leukocyte reactions: A possible mechanism for the generation of genetic diversity. Proceedings of the National Academy of Sciences, USA 83:6834-6838.

2. Bost, K.L., E.M. Smith, L.B. Wear and J.E. Blalock. 1987. Presence of ACTH and its receptor on a B lymphocytic cell line: A possible autocrine function for a neuroendocrine hormone. J Biological Reg Homeostatic Agents 1:23-28.

3. Harbour, D.V., E.M. Smith and J.E. Blalock. 1987. Splenic lymphocyte production of an endorphin during endotoxic shock. Brain Behavior Immun. 1:123-133.

4. Johnson, E.W. and E.M. Smith. 1987. Leukocyte production and sensitivity to neuroendocrine hormones induced by infectious agents or their products. In: Immunopharmacology of Infectious Diseases: Vaccine Adjuvants and Modulators of Non-specific Resistance. (eds. Majde, J. A.) Alan R. Liss, Inc., New York, pp 61-73.

5. Smith, E.M. and J.E. Blalock. 1987. Interactions between the interferon and endocrine systems. In: The Interferon System a Current Review to 1987. (eds. Baron, S., F. Dianzani, G.J. Stanton and W.R. Fleischmann), U. Texas Press, Austin, pp 253-260.

6. Smith, E.M., P. Brosnan, W.J. Meyer, III and J.E. Blalock. 1987. A corticotropin (ACTH) receptor on human mononuclear leukocytes: correlation with adrenal ACTH receptor activity, N Engl. J. Med. 317:1266-1269.

7. Harbour, D.V., E.M. Smith and J.E. Blalock. 1987. Novel processing pathway of proopiomelanocortin in lymphocytes: Endotoxin induction of a new prohormone-cleaving enzyme. J. Neuroscience Res. (In press).

8. Smith, E.M. and J.E. Blalock. 1987. A molecular basis for interactions between the immune and neuroendocrine systems. Internat. J. Neurosci (In Press).

9. Smith, E.M., D.V. Harbour and J.E. Blalock. 1987. Leukocyte production of endorphins. Ann. N.Y. Acad. Sci. (In Press).

10. Johnson, E.W., K.L. Bost, E.M. Smith and J.E. Blalock. 1987. Distribution of ACTH receptors on murine leukocytes. (Submitted to J. Immunol).

11. Harbour, D.V., Anderson, A., Farrington, J., Wassef, A., Smith, E.M., and Meyer, W.J. 1987. Decreased lymphocyte TSH responsiveness in patients with major depression. Biol. Psychiatry (In Press).

12. Smith, E.M. 1987. Hormonal activities of lymphokines, monokines, and other cytokines. Prog. Allergy (In Press).

13. Wassef, A., E.M. Smith, R.M. Rose, R. Gardner, H. Nguyen, and W.J. Meyer, III. 1987. Cellular aspects of glucocorticoid resistance: Preliminary suggestion of a qualitative or post-receptor function defect (Submitted to Biol. Psychiatr.)

14. Smith, E.M., Harbour, D.V., Coppenhaver, D.L., and Blalock, J.E. 1987. Amino acid sequence analysis of lymphocyte-derived ACTH (In preparation).

Abstracts (Year 1 of renewal):

1. Johnson, E.W., K.L. Bost, E.M. Smith and J.E. Blalock. 1986. Distribution and modulation of ACTH receptors on murine mononuclear leukocytes. Abstr. Sixth International Congress of Immunology.

2. Smith, E.M. 1986. A molecular basis for interactions between the immune and neuroendocrine systems. Abstr. 2nd International Workshop on Neuroimmunomodulation.

3. Smith, E.M. 1986. Leukocyte production of endogenous opiates and the presence of a functional opiate receptor. Abstr. 2nd International Workshop on Neuroimmunomodulation.

4. Wassef, A., Meyer, W.J., E.M. Smith, T. McCarty, E.B. Thompson and R.M. Rose. 1986. Leukocyte glucocorticoid related events in major depression: evidence for a partial post-receptor binding defect. Abstr. American College of Neuropsychopharmacology.

5. Johnson, E.W., G.R. Klimpel and E.M. Smith. 1987. Adrenocorticotropin (ACTH) receptors on murine leukocytes: Expression and association with cell-mediated cytotoxicity. Abstr. Fed. Amer. Soc. Exp. Biol..

6. Harbour, D., E.M. Smith and J.E. Blalock. 1987. Splenic lymphocyte production and processing of an endorphin component of endotoxic shock. Abstr. Fed. Amer. Soc. Exp. Biol..

7. Kruger, T.E., E.M. Smith and J.E. Blalock. 1987. Thyrotropin releasing hormone (TRH) enhancement of in vitro antibody production. Abstr. Fed. Amer. Soc. Exp. Biol..

8. Wear, L.B., K.L. Bost, E.M. Smith and J.E. Blalock. 1987. The presence of ACTH and its receptor on a B lymphocytic cell line. Abstr. Fed. Amer. Soc. Exp. Biol..

9. Johnson, E.W., G.R. Klimpel and E.M. Smith. 1987. Expression of adrenocorticotropin (ACTH) receptors on murine leukocytes and association with cell-mediated cytotoxicity. Abstr. Nat'l. Student Res. Forum.

10. Anderson, A., J. Farrington, D. Harbour, A. Wassef, E.M. Smith and W.J. Meyer. 1987. Decreased lymphocyte TSH responsiveness in patients with major depression. Abstr. Nat'l. Student Res. Forum.

11. Meyer, W.J., E.M. Smith, G.E. Richards, N.G. Greger, P.G. Brosnan and B.S. Keenan. 1987. ACTH receptor defect in adrenoleukodystrophy (ALD). Abstr. Amer. Pediatric Society and Society for Pediatric Research.

12. Harbour, D., E.M. Smith and W.J. Meyer. 1987. Molt 4 T-lymphoblastic leukemia cell production of immunoreactive TSH. Abst. Submitted to the Endocrine Society.

13. Harbour, D., Anderson, A., Farrington, J., Wassef, A., Smith, E.M., and Meyer, W.J. 1987. Decreased mononuclear leukocyte TSH secretion in patients with major depression. Abstr. Society for Neuroscience.

14. Smith, E.M., Richards, G.E., Greger, N.G., Jazayeri, A., Brosnan, P.G., Keenan, B.S., and Meyer, W.J. 1987. ACTH receptor defect in adrenoleukodystrophy (ALD). Abstr. Society for Neuroscience.

15. Smith, E.M., Harbour, D.V., and Blalock, J.E. 1987. Lymphocyte production of proopiomelanocortin: Processing and amino acid sequence of the polypeptide. Abstr. J. Neuroimmunol.

V. Literature Cited

1. Blalock, J.E., Bost, K.L. and Smith, E.M. 1985. J. Neuroimmunol. 10:31.

2. Blalock, J.E. and Smith, E.M. 1985. Immunology Today 6:1.

3. Smith, E.M., Morrill, A.C., Meyer, W.J. and Blalock, J.E. 1986. Nature 321:881.

4. Harbour-McMenamin, D., Smith, E.M. and Blalock, J.E. 1985. Infect, Immunol 48:813.

5. Harbour, D.V., Smith, E.M. and Blalock, J.E. 1987. J. Neurosci. Res. (In Press).

6. Uhler, M. and Herbert, E. 1983. J. Biol. Chem. 258:257-261.

7. Harbour-McMenamin, D.V., Smith, E.M. and Blalock, J.E. ????. P.N.A.S., USA 83:6834-6838.

8. Smith, E.M., Phan, M., Kruger, T.E., Coppenhaver, D.H. and Blalock, J.E. ????. P.N.A.S., USA 80:6010-6013.

9. Harbour, D.V., Anderson, A., Farrington, J., Wassef, A., Smith, E.M. and Meyer, W.J. 1987. J. Biol. Psych. (In Press).

Figure 1.

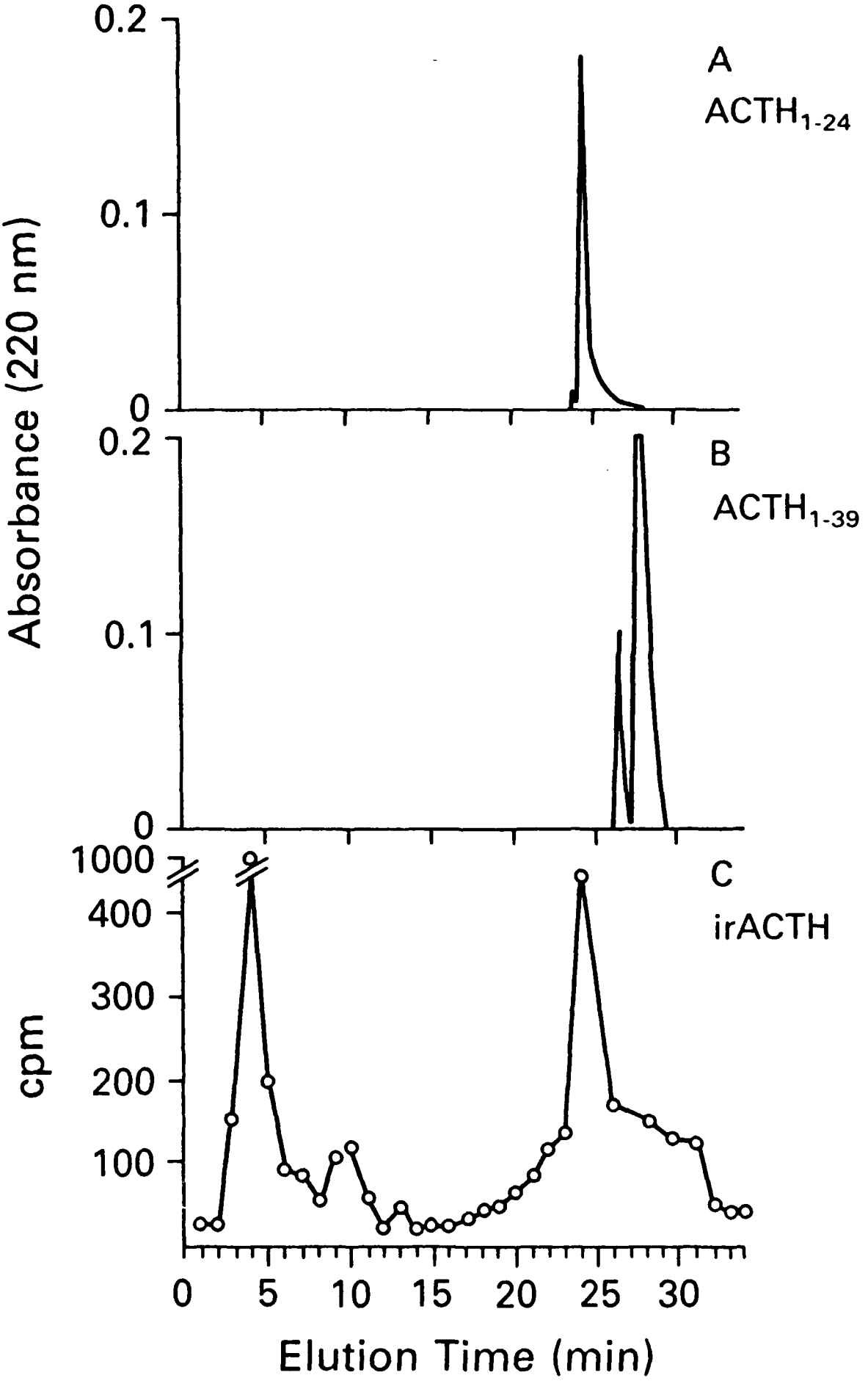
0.2
0.1
0
A
ACTH1-24
Absorbance (220 nm)
0.2
0.1
0
B
ACTH1-39
1000
400
300
200
100
cpm
C
irACTH
0
5
10
15
20
25
30
Elution Time (min)

Figure 2

Amino Acid Sequence Comparison of Mouse

Pituitary* and Leukocyte ACTH(1-39)

	1				5					10					15			
Pituitary	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val	Gly	Lys	Lys		
Leukocyte	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val	Gly	Lys	Lys		
					20					25					30			
	Arg	Arg	Pro	Val	Lys	Val	Tyr	Pro	Asn	Val	Ala	Glu	Asn	Gly	Ser	Ala		
	Arg	Arg	Pro	Val	Lys	Val	Tyr	Pro	Asn	Val	Ala	Glu	-	Gly	Leu	-		
			35				39											
	Glu	Ala	Phe	Pro	Leu	Glu	Phe											
	Glu	Ala	Phe	Pro	-	Glu	Phe											

*Uhler & Herbert J.B.C. 258:257(1983).

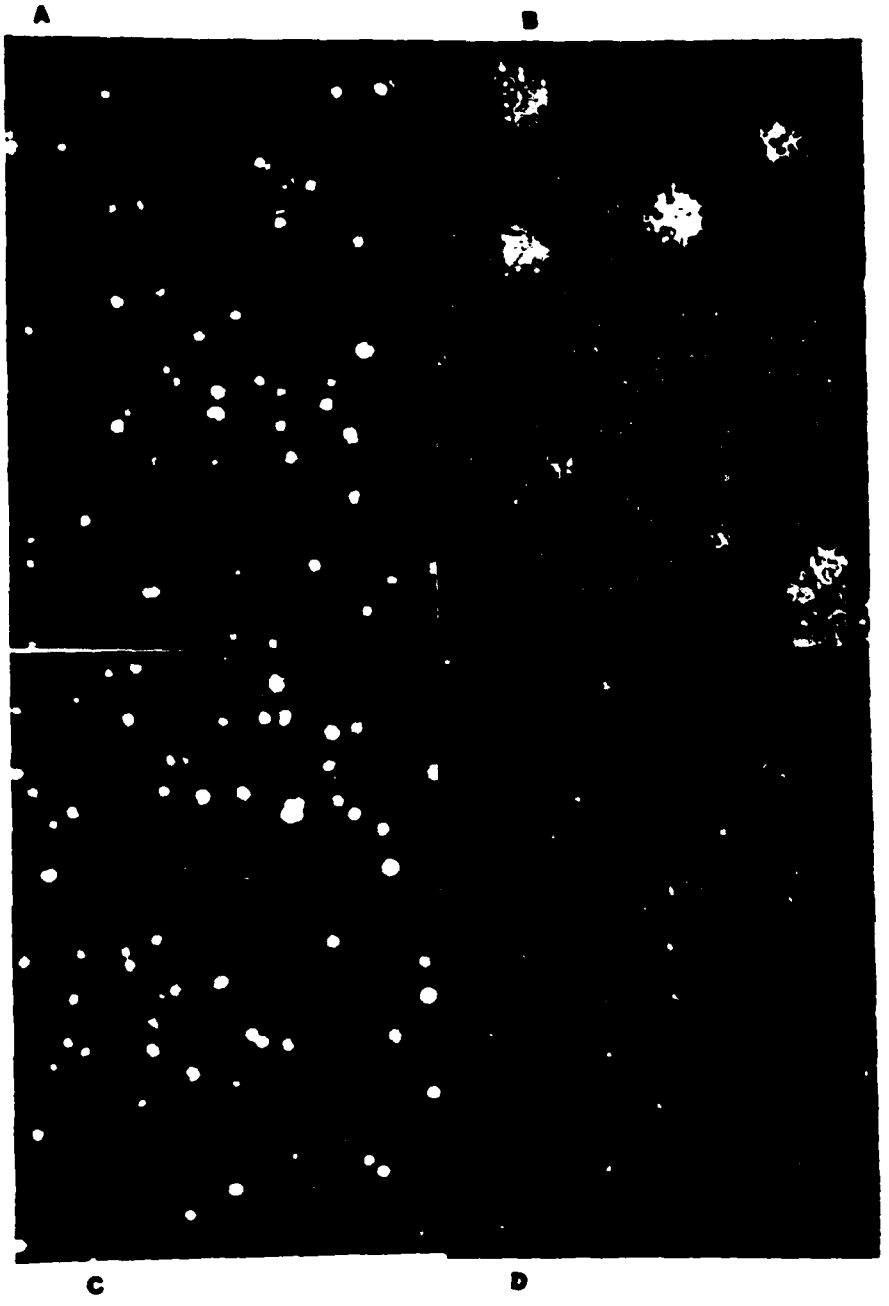

Figure 3. Reverse hemolytic plaque assy Molt 4 cells.

Sheep red blood cells were coupled to staph proten A and 12% suspension was mixed with equal volumes of Molt 4 cells. Antibody to TSH-β and guinea pig complement were added to final dilutions of 1:100 and 1:50, respectively. Sampels were loaded into glass slide chambers and plaques formed in humidity chambers at 37°C and were quantitated on 10 low power (10X) fields. A represents SEA stimulated Molt 4 cells (4X magnification), B represents high power view of SEA treated plaques (40X magnification), C is TRH stimulated Molt 4 cells (4X magnification), D is an anitbody control (4X magnification).

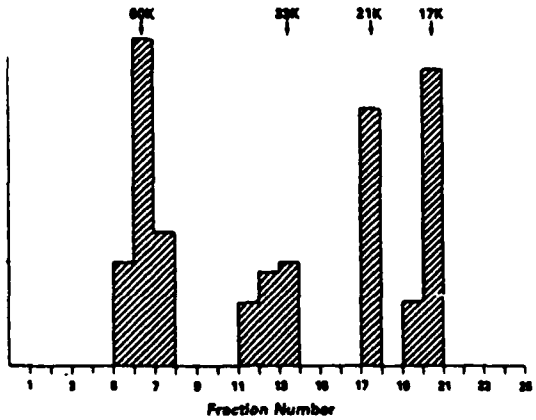

Figure 4. Polyacrylamide Gel Electrophoresis of Intrinsically Labeled ir-TSH.

Molecular weight standards are phosphorylase B (92K), bovine serum albumin (66K), ovalbumin (45K), carbonic anbydrase (31K), soybean trypsin inhibitor (21K) and lysozyme (14K). Molecular weights of ir-TSH were determined by log plot of molecular weight standards.

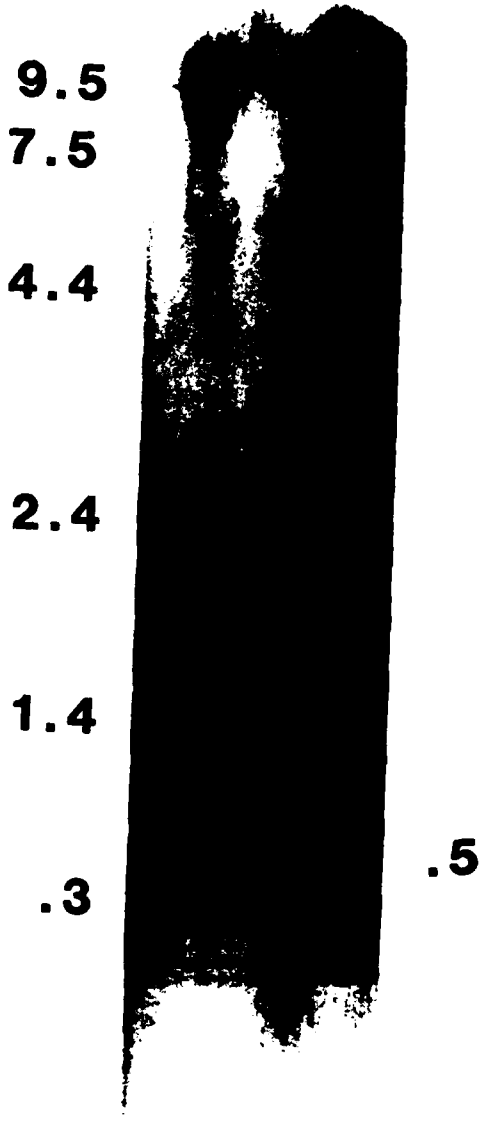

Figure 5. Northern Blot Analysis of Total RNA from Molt 4 Cells.

Molt 4 cells were induced with TRH for 2, 4, 6 or 24 hours, total RNA extracted, denatured and 25 µg/lane electrophored onto 1.5% agarose gels. The RNA was transferred to nitrocellulose filters and probed with P^{32} nick translated CDNA to TSH-β. From the left lane 7 is 2 hour TRH induction, lane 2 is 4 hour, lane 3 is 6 hour, lane 4 is 24 hour induction. Molecular weight markers are a log plot of an RNA ladder (Bethesda Research Labs).

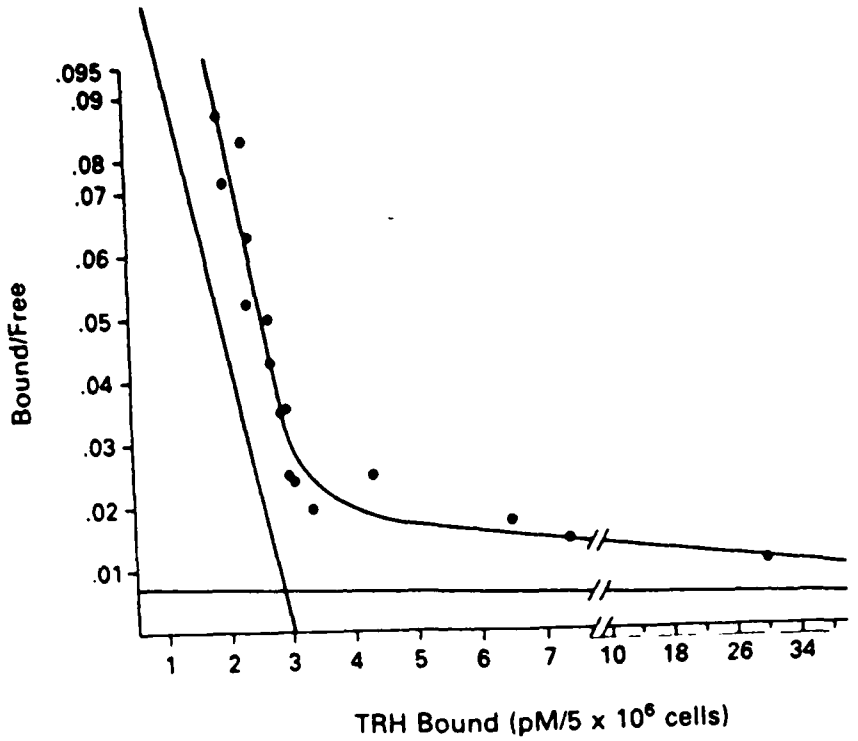

Figure 6. Scatchard Analysis of TRH Binding Studies on Molt 4 Cells.

$5X10^6$ Molt 4 cells were assayed for ability to binding ^3H-TRH (New England Nuclear specific activity - 38 Ci/mmol). Unbound or free radiolabeled TRH was determined by concentration of 10 µl aliquots of each dilution after binding assay was completed. Bound portion was determined as the concentration of radiolabeled TRH that remained bound to Molt 4 cells after extensive washing. Binding curves were resolved by method of Scatchard and each line was drawn by graphical method (123).

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INVESTIGATORS

Dr. Itamar B. Abrass
Department of Medicine
University of Washington
Harborview Medical Center
Seattle, WA 98104

Dr. Prince K. Arora
NICHD, Bldg 6, Room 132
National Institutes of Health
Bethesda, MD 20892

Dr. Karen Bulloch
Helicon Foundation
4622 Sante Fe Street
San Diego, CA 92109

Dr. Michael D. Cahalan
Department of Physiology and Biophysics
University of California, Irving
Irvine, CA 92717

Dr. Donald A. Chambers
Health Sciences Center
University of Illinois at Chicago
P.O. Box 6998
Chicago, IL 60680

Dr. Christopher L. Coe
Department of Psychology
Harlow Primate Laboratory
University of Wisconsin
Madison, WI 53715

Dr. Walla L. Dempsey
Department of Microbiology and Immunology
The Medical College of Pennsylvania
3300 Henry Avenue
Philadelphia, PA 19129

Dr. Adrian J. Dunn
Department of Neurescience
University of Florida
College of Medicine
Gainsville, FL 32610

Dr. David L. Felten
Department of Anatomy
University of Rochester
School of Medicine
601 Elmwood Avenue
Rochester, NY 14642

Dr. John F. Hansbrough
Department of Surgery
UCSD Medical Center
225 Dickinson Street
San Diego, CA 92103

Dr. William F. Hickey
Neuropathology Laboratories
454 Johnson Pavilion
University of Pennsylvania
Philadelphia, PA 19104

Dr. Robert L. Hunter
Department of Pathology
Emory Univ. School of Medicine
WMB 760
Atlanta, GA 30322

Dr. Terry C. Johnson
Division of Biology
Ackert Hall
Kansas State University
Manhattan, KS 66506

Dr. Sandra Levy
University of Pittsburgh
School of Medicine
3811 O'Hara Street
Pittsburgh, PA 15213

Dr. Lester Luborsky
Department of Psychiatry
308 Piersol Building/G1
University of Pennsylvania Hospital
Philadelphia, PA 19104

Dr. Steven F. Maier
Department of Psychology
University of Colorado
Campus Box 345
Boulder, CO 80309

Dr. Michael H. Melner
Department of Biochemistry
Univ of Miami School of Medicine
1600 N.W. 10th Avenue
Miami, FL 33136

Dr. Vera B. Morhenn
Department of Dermatology
Stanford University Medical School
Stanford, CA 94305

Dr. Jose R. Perez-Polo
Gail Borden Bldg., Rm., 436
University of Texas Medical Branch
Galveston, TX 77550-2777

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Department of Biological Sciences
Wayne State University
Detroit, MI 48202

Dr. Bruce S. Rabin
Clinical Immunopathology
Childrens Hospital
University of Pittsburgh Sch of Medicine
Pittsburgh, PA 15213

Dr. Seymour Reichlin
Director, Clinical Study Unit
New England Midical Center Hospitals, Inc.
171 Harrison Avenue
Boston, MA 02111

Dr. Eric M. Smith
Department of Psychiatry
University of Texas Medical Branch
Galveston, TX 77550

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